PATENT APPLICATION

METHODS OF GENETIC ANALYSIS USING NUCLEIC ACID PROBES

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PATENT

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METHODS OF GENETIC ANALYSIS USING NUCLEIC ACID ARRAYS

CROSS REFERENCE TO RELATED APPLICATIONS

The present application is a non-provisional application claiming priority from Provisional U.S. Patent Application Serial No. 60/100,678 filed September 17, 1998.

BACKGROUND OF THE INVENTION

The present invention provides a unique pool of nucleic acid sequences useful for analyzing molecular interactions of biological interest. The invention therefore relates to diverse fields impacted by the nature of molecular interaction, including chemistry, biology, medicine, and medical diagnostics.

FIELD OF THE INVENTION

Many biological functions are carried out by regulating the expression levels of various genes, either through changes in levels of transcription (*e.g.* through control of initiation, provision of RNA precursors, RNA processing, *etc.*) of particular genes, through changes in the copy number of the genetic DNA, or through changes in protein synthesis. For example, control of the cell cycle and cell differentiation, as well as diseases, are characterized by the variations in the transcription levels of a group of genes.

Gene expression is not only responsible for physiological functions, but also associated with pathogenesis. For example, the lack of sufficient functional tumor suppressor genes and/or the over expression of oncogene/protooncogenes leads to tumorgenesis. (See, e.g., Marshall, *Cell*, 64: 313-326 (1991) and Weinberg, Science, 254: 1138-1146 (1991.)) Thus, changes in the

expression levels of particular genes (*e.g.* oncogenes or tumor suppressors), serve as signposts for the presence and progression of various diseases.

As a consequence, novel techniques and apparatus are needed to study gene expression in specific biological systems.

All documents, i.e., publications and patent applications, cited in this disclosure, including the foregoing, are incorporated by reference herein in their entireties for all purposes to the same extent as if each of the individual documents were specifically and individually indicated to be so incorporated by reference herein in its entirety.

SUMMARY OF THE INVENTION

The invention provides nucleic acid sequences which are complementary to particular genes and makes them available for a variety of analyses, including, for example, gene expression analysis. For example, in one embodiment the invention comprises an array comprising of any 10 or more, 100 or more, 1000, or more, 10,000 or more or 100,000 or more nucleic acid probes containing 9 or more consecutive nucleotides from the sequences listed in SEQ ID NOS: 1 -127811, or the perfect match, perfect mismatch, antisense match or antisense mismatch thereof. In a further embodiment, the invention comprises the use of any of the above arrays or fragments disclosed in TABLE 1 to: monitor gene expression levels by hybridization of the array to a DNA library; monitor gene expression levels by hybridization to an mRNA-protein fusion compound; identify polymorphisms; identify biallelic markers; produce genetic maps; analyze genetic variation; comparatively analyze gene expression between different species; analyze gene knockouts; or, to hybridize tag-labeled compounds. In a further embodiment the invention comprises a method of analysis comprising of hybridizing one or

more pools of nucleic acids to two or more of the fragments disclosed in TABLE 1 and detecting said hybridization. In a further embodiment the invention comprises the use of any one or more of the fragments disclosed in TABLE 1 as a primer for PCR. In a further embodiment the invention comprises the use of any one or more of the fragments disclosed in TABLE 1 as a ligand.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

Massive Parallel Screening: The phrase "massively parallel screening" refers to the simultaneous screening of at least about 100, preferably about 1000, more preferably about 10,000 and more preferably about 100,000 different nucleic acid hybridizations.

Nucleic Acid: The terms "nucleic acid" or "nucleic acid molecule" refer to a deoxyribonucleotide or ribonucleotide polymer in either single-or double-stranded form, and unless otherwise limited, would encompass analogs of natural nucleotides that can function in a similar manner as naturally occurring nucleotides. Nucleic acids may be derived from a variety or sources including, but not limited to, naturally occurring nucleic acids, clones, synthesis in solution or solid phase synthesis.

Probe: As used herein a "probe" is defined as a nucleic acid capable of binding to a target nucleic acid of complementary sequence through one or more types of chemical bonds, usually through complementary base pairing, usually through hydrogen bond formation. As used herein, a probe may include natural (*i.e.* A, G, U, C, or T) or modified bases (7-deazaguanosine,

inosine, *etc.*). In addition, the bases in probes may be joined by a linkage other than a phosphodiester bond, so long as it does not interfere with hybridization. Thus, probes may be peptide nucleic acids in which the constituent bases are joined by peptide bonds rather than phosphodiester linkages.

Target nucleic acid: The term "target nucleic acid" or "target sequence" refers to a nucleic acid or nucleic acid sequence which is to be analyzed. A target can be a nucleic acid to which a probe will hybridize. The probe may or may not be specifically designed to hybridize to the target. It is either the presence or absence of the target nucleic acid that is to be detected, or the amount of the target nucleic acid that is to be quantified. The term target nucleic acid may refer to the specific subsequence of a larger nucleic acid to which the probe is directed or to the overall sequence (*e.g.*, gene or mRNA) whose expression level it is desired to detect. The difference in usage will be apparent from context.

mRNA or transcript: The term "mRNA" refers to transcripts of a gene. Transcripts are RNA including, for example, mature messenger RNA ready for translation, products of various stages of transcript processing. Transcript processing may include splicing, editing and degradation.

Subsequence: "Subsequence" refers to a sequence of nucleic acids that comprise a part of a longer sequence of nucleic acids.

Perfect match: The term "match," "perfect match," "perfect match probe" or "perfect match control" refers to a nucleic acid that has a sequence that is perfectly complementary to a particular target sequence. The nucleic acid is typically perfectly complementary to a portion (subsequence) of the target sequence. A perfect match (PM) probe can be a "test probe", a

"normalization control" probe, an expression level control probe and the like. A perfect match control or perfect match is, however, distinguished from a "mismatch" or "mismatch probe."

Mismatch: The term "mismatch," "mismatch control" or "mismatch probe" refers to a nucleic acid whose sequence is deliberately selected not to be perfectly complementary to a particular target sequence. As a non-limiting example, for each mismatch (MM) control in a high-density probe array there typically exists a corresponding perfect match (PM) probe that is perfectly complementary to the same particular target sequence. The mismatch may comprise one or more bases. While the mismatch(es) may be located anywhere in the mismatch probe, terminal mismatches are less desirable because a terminal mismatch is less likely to prevent hybridization of the target sequence. In a particularly preferred embodiment, the mismatch is located at or near the center of the probe such that the mismatch is most likely to destabilize the duplex with the target sequence under the test hybridization conditions. A homo-mismatch substitutes an adenine (A) for a thymine (T) and vice versa and a guanine (G) for a cytosine (C) and vice versa. For example, if the target sequence was: AGGTCCA, a probe designed with a single homo-mismatch at the central, or fourth position, would result in the following sequence: TCCTGGT.

Array: An "array" is a solid support with at least a first surface having a plurality of different nucleic acid sequences attached to the first surface.

Gene Knockout: the term "gene knockout, " as defined in Lodish et al *Molecular Cell Biology 3rd Edition*, Scientific American Books pub., which is hereby incorporated in its entirety for all purposes is, is a technique for selectively inactivating a gene by replacing it with a mutant allele in an otherwise normal organism.

DNA Library - as used herein the term "genomic library" or "genomic DNA library" refers to a collection of cloned DNA molecules consisting of fragments of the entire genome (genomic library) or of DNA copies of all the mRNA produced by a cell type (cDNA library) inserted into a suitable cloning vector.

Polymorphism - "polymorphism" refers to the occurrence of two or more genetically determined alternative sequences or alleles in a population. A polymorphic marker or site is the locus at which divergence occurs. Preferred markers have at least two alleles, each occurring at a frequency of greater than 1%, and more preferably greater than 10% or 20% of the selected population. A polymorphic locus may be as small as one base pair. Polymorphic markers include restriction fragment length polymorphisms, variable number or tandem repeats (VNTR's), hypervariable regions, minisatellites, dinucleotide repeats, trinucleotide repeats, tetranucleotide repeats, simple sequence repeats, and insertion elements such as ALU. The first identified allelic form is arbitrarily designated as the reference form and other allelic forms are designated as alternative or variant alleles. The allelic form occurring most frequently in a selected population is sometimes referred to as the wildtype form. Diploid organisms may be homozygous or heterozygous for allelic forms. A diallelic or biallelic polymorphism has two forms. A triallelic polymorphism has three forms.

Genetic map - a "genetic map" is a map which presents the order of specific sequences on a chromosome.

Genetic variation - "genetic variation" refers to variation in the sequence of the same region between two or more organisms.

Hybridization - the association of two complementary nucleic acid strands or their derivatives (such as PNA) to form double stranded molecules. Hybrids can contain two DNA strands, two RNA strands, or one DNA and one RNA strand.

mRNA-protein fusion - a compound whereby an mRNA is directly attached to the peptide or protein it incodes by a stable covalent linkage.

Ligand - any molecule, other than an enzyme substrate, that binds tightly and specifically to a macromolecule, for example, a protein, forming a macromolecule-ligand complex.

II. General

TABLE 1, encompassed in Appendix I, presents target sequences included in the invention. Each target sequence from columns 2,5 and 8 corresponds to and represents at least four additional nucleic acid sequences included in the invention. For example, if the first nucleic acid sequence listed in TABLE 1 is: **cgccggaaagcgtgccaatcaacat** the additional sequences included in the invention which are represented by this nucleic acid sequence are, for example, :

gcggcctttcgcacggccagttgta = (perfect) sense match

gcggcctttcgctcggccagttgta = sense mismatch

tacaactggccgagcgaaaggccgc = (perfect) antisense match

tacaactggccgtgcgaaaggccgc = antisense mismatch

Accordingly, for each nucleic acid sequence listed in TABLE 1, this disclosure includes the corresponding sense match, sense mismatch, antisense match and antisense mismatch. The

position of the mismatch is not limited to the above example, it may be located anywhere in the nucleic acid sequence and may comprise one or more bases.

Consequently, the present invention includes: a) the target sequences listed in TABLE 1, columns 2, 5 and 8 or the sense-match, sense mismatch, antisense match or antisense mismatch thereof; b) clones which comprise the target nucleic acid sequences listed in TABLE 1, columns 2, 5 and 8 or the sense-match, sense mismatch, antisense match or antisense mismatch thereof; c) longer nucleotide sequences which include the nucleic acid sequences listed in TABLE 1, columns 2, 5, and 8 or the sense-match, sense mismatch, antisense match or antisense mismatch thereof and d) subsequences greater than 9 nucleotides in length of the target nucleic acid sequences listed in TABLE 1, columns 2, 5, and 8 or the sense match, sense mismatch, antisense match or antisense mismatch.

Target sequences were chosen from clusters of known murine genes available on the Unigene database as of August 15, 1996. Target sequences were selected using the computer based methods described in US patent application No. 08/772,376 incorporated herein by reference for all purposes.

For each target sequence listed in TABLE 1 is a corresponding genbank database accession number. These accession numbers allow for the identification of sequences located in the genbank sequence database through the use of computer programs such as BLAST. Access to BLAST is available to the public through the internet at, for example, http://www.ncbi.nim.nih.gov. One of skill in the art will be familiar with the use of the BLAST program to obtain information about particular sequences in order to, for example, determine the species from which the sequence is derived, determine the gene from which the sequence is

derived, to determine other genes and species which contain similar sequences and to determine the degree of similarity between one sequence and another. All information relating to the target sequences available through the genbank database is hereby incorporated by reference for all purposes.

The present invention provides a pool of unique nucleotide sequences complementary to murine genes and ESTs in particular embodiments which alone, or in combinations of 2 or more, 10 or more, 100 or more, 1,000 or more, 10,000 or more, or 100,000 or more, can be used for a variety of applications.

In one embodiment, the present invention provides for a pool of unique nucleotide sequences which are complementary to approximately 6500 murine genes formed into a high density array of probes suitable for array based massive parallel gene expression. Array based methods for monitoring gene expression are disclosed and discussed in detail in U.S. Patent No. Applications, 08/670,118, and 08/772,376 and PCT Application WO 92/10588 (published on June 25, 1992), all of which are incorporated herein by reference for all purposes. Generally those methods of monitoring gene expression involve (1) providing a pool of target nucleic acids comprising RNA transcript(s) of one or more target gene(s), or nucleic acids derived from the RNA transcript(s); (2) hybridizing the nucleic acid sample to a high density array of probes and (3) detecting the hybridized nucleic acids and calculating a relative expression (transcription, RNA processing or degradation) level.

The development of Very Large Scale Immobilized Polymer Synthesis or VLSIPS™ technology has provided methods for making very large arrays of nucleic acid probes in very small arrays. See U.S. Patent No. 5,143,854 and PCT patent publication Nos. WO 90/15070

and 92/10092, and Fodor *et al.*, *Science*, 251, 767-77 (1991), each of which is incorporated herein by reference. U.S. Patent application Serial No. 08/670,118, describes methods for making arrays of nucleic acid probes that can be used to detect the presence of a nucleic acid containing a specific nucleotide sequence. Methods of forming high density arrays of nucleic acids, peptides and other polymer sequences with a minimal number of synthetic steps are known. The nucleic acid array can be synthesized on a solid substrate by a variety of methods, including, but not limited to, light-directed chemical coupling, and mechanically directed coupling.

In a preferred detection method, the array of immobilized nucleic acids, or probes, is contacted with a sample containing target nucleic acids, to which a flourescent label is attached. Target nucleic acids hybridize to the probes on the array and any non-hybridized nucleic acids are removed. The array containing the hybridized target nucleic acids are exposed to light which excites the flourescent label. The resulting flourescent intensity, or brightness, is detected. Relative brightness is used to determine which probe is the best candidate for the perfect match to the hybridized target nucleic acid because flourescent intensity (brightness) corresponds to binding affinity. Once the position of the perfect match probe is known, the sequence of the hybridized target nucleic is known because the sequence and position of the probe is known.

In the array of the present invention the probes are presented in pairs, one probe in each pair being a perfect match to the target sequence and the other probe being identical to the perfect match probe except that the central base is a homo-mismatch. Mismatch probes provide a control for non-specific binding or cross-hybridization to a nucleic acid in the sample other

than the target to which the probe is directed. Thus, mismatch probes indicate whether a hybridization is or is not specific. For example, if the target is present, the perfect match probes should be consistently brighter than the mismatch probes because fluorescence intensity, or brightness, corresponds to binding affinity. (See, for example US Patent No. 5,324,633, which is incorporated herein for all purposes.) In addition, if all central mismatches are present, the mismatch probes can be used to detect a mutation. Finally the difference in intensity between the perfect match and the mismatch probe (I(PM)-I(MM)) provides a good measure of the concentration of the hybridized material. See pending PCT application No. 98/11223, which is incorporated herein by reference for all purposes. The probe pairs are presented in both sense and antisense orientation, thereby eliciting a total of four probes per target sequence: sense match, sense mismatch, antisense match and antisense mismatch.

In another embodiment, the current invention provides a pool of sequences which may be used as probes for their complementary genes listed in the genbank database. Methods for making probes are well known. See for example Sambrook, Fritsche and Maniatis. "Molecular Cloning A laboratory Manual" 2nd Ed. Cold Spring Harbor Press (1989) ("Maniatis et al.") which is hereby incorporated in its entirety by reference for all purposes. Maniatis et al. describes a number of uses for nucleic acid probes of defined sequence. Some of the uses described by Maniatis et al. include: to screen cDNA or genomic DNA libraries, or subclones derived from them, for additional clones containing segments of DNA that have been isolated and previously sequenced; in Southern, northern, or dot-blot hybridization to identify or detect the sequences of specific genes; in Southern, or dot-blot hybridization of genomic DNA to detect specific mutations in genes of known sequence; to detect specific mutations generated by

site-directed mutagenesis of cloned genes; and to map the 5' termini of mRNA molecules by primer extensions. Maniatis et al. describes other uses for probes throughout. See also Alberts et al. *Molecular Biology of the Cell 3rd edition,* Garland Publishing Inc. (1994) p. 307 and Lodish et al. *Molecular Cell Biology, 3rd edition,* Scientific American Books (1995) p. 285-286, each of which is hereby incorporated by reference in its entirety for all purposes, for a brief discussion of the use of nucleic acid probes in *in situ hybridization*. Other uses for probes derived from the sequences disclosed in this invention will be readily apparent to those of skill in the art. See, for example, Lodish et al. *Molecular Cell Biology, 3rd edition,* Scientific American Books (1995) p.229-233, incorporated above, for a description of the construction of genomic libraries.

In another embodiment, the current invention may be combined with known methods to monitor expression levels of genes in a wide variety of contexts. For example, where the effects of a drug on gene expression are to be determined, the drug will be administered to an organism, a tissue sample, or a cell and the gene expression levels will be analyzed. For example, nucleic acids are isolated from the treated tissue sample, cell, or a biological sample from the organism and from an untreated organism tissue sample or cell, hybridized to a high density probe array containing probes directed to the gene of interest and the expression levels of that gene are determined. The types of drugs that may be used in these types of experiments include, but are not limited to, antibiotics, antivirals, narcotics, anti-cancer drugs, tumor suppressing drugs, and any chemical composition which may affect the expression of genes *in vivo* or *in vitro*. The current invention is particularly suited to be used in the types of analyses described by, for example, pending US Applications No. 08/772,376 and PCT Application No. 98/11223, each of

which is incorporated by reference in its entirety for all purposes. As described in Wodicka et al., Nature Biotechnology 15 (1997), (hereby incorporated by reference in its entirety for all purposes), because mRNA hybridization correlates to gene expression level, hybridization patterns can be compared to determine differential gene expression. As non-limiting examples: hybridization patterns from samples treated with certain types of drugs may be compared to hybridization patterns from samples which have not been treated or which have been treated with a different drug; hybridization patterns for samples infected with a specific virus may be compared against hybridization patterns from non-infected samples; hybridization patterns for samples with cancer may be compared against hybridization patterns for samples without cancer; hybridization patterns of samples from cancerous cells which have been treated with a tumor suppressing drug may be compared against untreated cancerous cells, etc. Zhang et al., Science 276 1268-1272, (hereby incorporated by reference in its entirety for all purposes), provides an example of how gene expression data can provide a great deal of insight into cancer research. One skilled in the art will appreciate that a wide range of applications will be available using 2 or more, 10 or more, 100 or more, 1000 or more, 10,000 or more or 100,000 or more of the Table 1 sequences as probes for gene expression analysis. The combination of the DNA array technology and the mouse specific probes in this disclosure is a powerful tool for studying gene expression.

In another embodiment, the invention may be used in conjunction with the techniques which link specific proteins to the mRNA which encodes the protein. (See for example Roberts and Szostak Proc. Natl, Acad. Sci. *94* 12297-12302 (1997) which is incorporated herein in its entirety for all purposes.) Hybridization of these mRNA-protein fusion compounds to arrays

comprised of 2 or more, 10 or more, 100 or more, 1000 or more, 10,000 or more, or 100,000 or more the sequences disclosed in the present invention provides a powerful tool for monitoring expression levels.

In one embodiment, the current invention provides a pool of unique nucleic acid sequences which can be used for parallel analysis of gene expression under selective conditions. Without wishing to be limited, genetic selection under selective conditions could include: variation in the temperature of the organism's environment; variation in pH levels in the organism's environment; variation in an organism's food (type, texture, amount etc.); variation in an organism's surroundings; etc. Arrays, such as those in the present invention, can be used to determine whether gene expression is altered when an organism is exposed to selective conditions.

Methods for using nucleic acid arrays to analyze genetic selections under selective conditions are known. (See for example, R. Cho et al., Proc. Natl. Acad. Sci. *95* 3752-3757 (1998) incorporated herein in its entirety for all purposes.) Cho et al. describes the use of a high-density array containing oligonucleotides complementary to every gene in the yeast *Saccharomyces cerevisiae* to perform two-hybrid protein-protein interaction screens for *S. cerevisiae* genes implicated in mRNA splicing and microtubule assembly. Cho et al. was able to characterize the results of a screen in a single experiment by hybridization of labeled DNA derived from positive clones. Briefly, as described by Cho et al., two proteins are expressed in yeast as fusions to either the DNA-binding domain or the activation domain of a transcription factor. Physical interaction of the two proteins reconstitutes transcriptional activity, turning on a gene essential for survival under selective conditions. In screening for novel protein-protein

interactions, yeast cells are first transformed with a plasmid encoding a specific DNA-binding fusion protein. A plasmid library of activation domain fusions derived from genomic DNA is then introduced into these cells. Transcriptional activation fusions found in cells that survive selective conditions are considered to encode peptide domains that may interact with the DNA-binding domain fusion protein. Clones are then isolated from the two-hybrid screen and mixed into a single pool. Plasmid DNA is purified from the pooled clones and the gene inserts are amplified using PCR. The DNA products are then hybridized to yeast whole genome arrays for characterization. The methods employed by Cho et al. are applicable to the analysis of a range of genetic selections. High density arrays created using two or more, 10 or more, 100 or more, 1000 or more, 10,000 or more, or 100,000 or more of the sequences disclosed in the current invention can be used to analyze genetic selections in the mouse system using the methods described in Cho et al.

In another embodiment, the current invention provides a pool of unique nucleic acid sequences which can be used to identify biallelic markers, providing a novel and efficient approach to the study of genetic variation. For example, methods for using high density arrays comprised of probes which are complementary to the genomic DNA of a particular species to interrogate polymorphisms are well known. (See for example, US Patent Application Nos. 08/965,620 and 08/853,370 which are hereby incorporated herein for all purposes.) Pools of 2 or more, 10 or more, 100 or more, 1000 or more, 10,000 or more, or 100,000 or more of the sequences disclosed in this invention combined with the methods described in the above patent applications provides a tool for studying genetic variation in the mouse system.

In another embodiment of the invention, genetic variation can be used to produce genetic maps of various strains of mouse. Winzeler et al., "Direct Allelic Variation Scanning of the Yeast Genome" Science (in press) (1998), which is hereby incorporated for all purposes describe methods for conducting this type of screening with arrays containing probes complementary to the yeast genome. Briefly, genomic DNA from strains which are phenotypically different are isolated, fragmented, and labelled. Each strain is then hybridized to identical arrays comprised of the nucleic acid sequences complementary to the system being studied. Comparison of hybridization patterns between the various strains then serve as genetic markers. As described by Winzler et al, these markers can then be used for linkage analysis. High density arrays created from 2 or more, 10 or more, 100 or more, 1000 or more, 10,000 or more, or 100,000 or more of the sequences disclosed in this invention can be used to study genetic variation using the methods described by Winzler et al.

In another embodiment, the present invention may be used for cross-species comparisons. One skilled in the art will appreciate that it is often useful to determine whether a gene present in one species, for example the mouse, is present in a conserved format in another species, including, without limitation, mouse, human, chicken, zebrafish, *drosophila*, or yeast. See, for example, Andersson et al., Mamm Genome 7(10):717-734 (1996,) which is hereby incorporated by reference for all purposes, which describes the utility of cross-species comparisons. The use of 2 or more, 10 or more, 100 or more, 1000 or more, 10,000 or more or 100,000 or more of the sequences disclosed in this invention in an array can be used to determine whether any of the sequence from one or more of the murine genes represented by the sequences disclosed in this invention is conserved in another species by, for example,

hybridizing genomic nucleic acid samples from another species to an array comprised of the sequences disclosed in this invention. Areas of hybridization will yield genomic regions where the nucleotide sequence is highly conserved between the interrogation species and the mouse.

In another embodiment, the present invention may be used to characterize the genotype of knockouts. Methods for using gene knockouts to identify a gene are well known. See for example, Lodish et al. *Molecular Cell Biology, 3rd Edition*, Scientific American Books pub pp. 292-296 and US Patent No. 5,679,523 which are hereby incorporated by reference for all purposes. By isolating genomic nucleic acid samples from knockout species with a known phenotype and hybridizing the samples to an array comprised of 2 or more, 10 or more, 100 or more, 1000 or more, 10,000 or more, or 100,000 or more of the sequences disclosed in this invention, candidates genes which contribute to the phenotype will be identified and made accessible for further characterization.

In another embodiment, the present invention may be used to identify new gene family members. Methods of screening libraries with probes are well known. (See, for example, Maniatis et al, incorporated by reference above.) Because the present invention is comprised of nucleic acid sequences from specific known genes, 2 or more, 10 or more, 100 or more, 1000 or more, 10,000 or more, or 100,000 or more of sequences disclosed in this invention may be used as probes to screen genomic libraries to look for additional family members of those genes from which the target sequences are derived.

In another embodiment, the present invention may be used to provide nucleic acid sequences to be used as tag sequences. Tag sequences are a type of genetic "bar code" which can be used to label compounds of interest. The analysis of deletion mutants using tag

sequences is described in, for example, Shoemaker et al., Nature Genetics 14 450-456 (1996), which is hereby incorporated by reference in its entirety for all purposes. Shoemaker et al. describes the use of PCR to generate large numbers of deletion strains. Each deletion strain is labelled with a unique 20-base tag sequence that can be hybridized to a high-density oligonucleotide array. The tags serve as unique identifiers (molecular bar codes) that allow analysis of large numbers of deletion strains simultanously through selective growth conditions. The use of tag sequences need not be limited to this example however. The utility of using unique known short oligonucleotide sequences capable of hybridizing to a nucleic acid array to label various compounds will be apparent to one skilled in the art. One or more, 10 or more, 100 or more, 1000 or more, 10,000 or more, or 100,000 or more of the Table 1 sequences are excellent candidates to be used as tag sequences.

In another embodiment of the invention, the sequences of this invention may be used to generate primers directed to their corresponding genes as disclosed in the Genbank or any other public database. These primers may be used in such basic techniques as sequencing or PCR, see for example Maniatis et al., incorporated by reference above.

In another embodiment, the invention provides a pool of nucleic acid sequences to be used as ligands for specific genes. The sequences disclosed in this invention may be used as ligands to their corresponding genes as disclosed in the Genbank or any other public database. Compounds which specifically bind known genes are of interest for a variety of uses. One particular clinical use is to act as an antisense protein which specifically binds and disables a gene which has been, for example, linked to a disease. Methods and uses for ligands to specific

genes are known. See for example, US Patent No. 5,723,594 which is hereby incorporated by reference in its entirety for all purposes.

In a preferred embodiment, the hybridized nucleic acids are detected by detecting one or more labels attached to the sample nucleic acids. The labels may be incorporated by any of a number of means well known to those of skill in the art. In one embodiment, the label is simultaneously incorporated during the amplification step in the preparation of the sample nucleic acids. Thus, for example, polymerase chain reaction (PCR) with labeled primers or labeled nucleotides will provide a labeled amplification product. In a another embodiment, transcription amplification, as described above, using a labeled nucleotide (*e.g.* fluorescein-labeled UTP and/or CTP) incorporates a label into the transcribed nucleic acids.

Alternatively, a label may be added directly to the original nucleic acid sample (*e.g.*, mRNA, polyA mRNA, cDNA, *etc.*) or to the amplification product after the amplification is completed. Means of attaching labels to nucleic acids are well known to those of skill in the art and include, for example nick translation or end-labeling (*e.g.* with a labeled RNA) by kinasing of the nucleic acid and subsequent attachment (ligation) of a nucleic acid linker joining the sample nucleic acid to a label (*e.g.*, a fluorophore).

Detectable labels suitable for use in the present invention include any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include biotin for staining with labeled streptavidin conjugate, magnetic beads (*e.g.*, Dynabeads™), fluorescent dyes (*e.g.*, fluorescein, texas red, rhodamine, green fluorescent protein, and the like), radiolabels (*e.g.*, ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P), phosphorescent labels, enzymes (*e.g.*, horse radish peroxidase, alkaline phosphatase and

others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic (*e.g.*, polystyrene, polypropylene, latex, etc.) beads. Patents teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241, each of which is hereby incorporated by reference in its entirety for all purposes.

Means of detecting such labels are well known to those of skill in the art. Thus, for example, radiolabels may be detected using photographic film or scintillation counters, fluorescent markers may be detected using a photodetector to detect emitted light. Enzymatic labels are typically detected by providing the enzyme with a substrate and detecting the reaction product produced by the action of the enzyme on the substrate, and colorimetric labels are detected by simply visualizing the colored label.

The label may be added to the target nucleic acid(s) prior to, or after the hybridization. So called "direct labels" are detectable labels that are directly attached to or incorporated into the target nucleic acid prior to hybridization. In contrast, so called "indirect labels" are joined to the hybrid duplex after hybridization. Often, the indirect label is attached to a binding moiety that has been attached to the target nucleic acid prior to the hybridization. Thus, for example, the target nucleic acid may be biotinylated before the hybridization. After hybridization, an aviden-conjugated fluorophore will bind the biotin bearing hybrid duplexes providing a label that is easily detected. For a detailed review of methods of labeling nucleic acids and detecting labeled hybridized nucleic acids see *Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 24: Hybridization With Nucleic Acid Probes*, P. Tijssen, ed. Elsevier, N.Y., (1993) which is hereby incorporated by reference in its entirety for all purposes.

Fluorescent labels are preferred and easily added during an *in vitro* transcription reaction. In a preferred embodiment, fluorescein labeled UTP and CTP are incorporated into the RNA produced in an *in vitro* transcription reaction as described above.

EXAMPLE

The following example serves to illustrate the type of experiment that could be conducted using the invention.

Expression Monitoring by Hybridization to High Density Oligonucleotide Arrays

Arrays containing the desired number of probes can be synthesized using the method described in US Patent No. 5,143,854, incorporated by reference above. Extracted poly $(A)^+$RNA can then be coverted to cDNA using the methods described below. The cDNA is then transcribed in the presence of labeled ribonucleotide triphosphates. The label may be biotin or a dye such as fluorescein. RNA is then fragmented with heat in the presence of magnesium ions. Hybridizations are carried out in a flow cell that contains the two-dimensional DNA probe arrays. Following a brief washing step to remove unhybridized RNA, the arrays are scanned using a scanning confocal microscope.

1. A method of RNA preparation:

Labeled RNA is prepared from clones containing a T7 RNA polymerase promoter site by incorporating labeled ribonucleotides in an IVT reaction. Either biotin-labeled or fluorescein-labeled UTP and CTP (1:3 labeled to unlabeled) plus unlabeled ATP and GTP is used for the reaction with 2500 U of T7 RNA polymerase. Following the reaction unincorporated nucleotide triphosphates are removed using size-selective membrane such as Microcon - 100, (Amicon, Beverly, MA). The total molar concentration of RNA is based on a measurement of the absorbance at 260 nm. Following quantitation of RNA amounts, RNA is fragmented randomly to an average length of approximately 50 bases by heating at 94° in 40 mM Tris-acetate pH 8.1, 100 mM potassium acetate, 30 mM magnesium acetate, for 30 to 40 min. Fragmentation reduces possible interference from RNA secondary structure, and minimizes the effects of multiple interactions with closely spaced probe molecules. For material

made directly from cellular RNA, cytoplasmic RNA is extracted from cells by the method of Favaloro et al. Methods Enzymol. 65:718-749 (1980) hereby incorporated by reference for all purposes, and poly $(A)^+$ RNA is isolated with an oligo dT selection step using, for example, Poly Atract, (Promega, Madison, WI). RNA can be amplified using a modification of the procedure described by Eberwine et al. Proc. Natl. Acad. Sci. USA 89:3010-3014 (1992) hereby incorporated by reference for all purposes. Microgram amounts of poly $(A)^+$ RNA are converted into double stranded cDNA using a cDNA synthesis kit (kits may be obtained from Life Technologies, Gaithersburg, MD) with an oligo dT primer incorporating a T7 RNA polymerase promoter site. After second-strand synthesis, the reaction mixture is extracted with phenol/chloroform, and the double-stranded DNA isolated using a membrane filtration step using, for example, Microcon -100, (Amicon). Labeled cRNA can be made directly from the cDNA pool with an IVT step as described above. The total molar concentration of labeled cRNA is determined from the absorbance at 260nm and assuming an average RNA size of 1000 ribonucleotides. The commonly used convention is that 1 OD is equivalent to 40 ug of RNA, and that 1 ug of cellular mRNA consists of 3 pmol of RNA molecules. Cellular mRNA may also be labeled directly without any intermediate cDNA synthesis steps. In this case, Poly $(A)^+$ RNA is fragmented as described, and the 5' ends of the fragments are kinased and then incubated overnight with a biotinylated oligoribonucleotide (5'-biotin-AAAAAA-3') in the presence of T4 RNA ligase (available from Epicentre Technologies, Madison, WI). Alternatively, mRNA has been labeled directly by UV-induced cross-linking to a psoralen derivative linked to biotin (available from Schleicher & Schuell, Keene, NH).

2. Array hybridization and Scanning:

Array hybridization solutions can be made containing 0.9 M NaCl, 60mM EDTA, and 0.005% Triton X-100, adjusted to pH 7.6 (referred to as 6xSSPE-T). In addition, the solutions should contain 0.5 mg/ml unlabeled, degraded herring sperm DNA (available from Sigma, St. Louis, MO). Prior to hybridization, RNA samples are heated in the hybridization solution to 99°C for 10 min, placed on ice for 5 min, and allowed to equilibrate at room temperature before being placed in the hybridization flow cell. Following hybridization, the solutions are removed, the arrays washed with 6xSSPE-T at 22C for 7 min, and then washed with 0.5xSSPE-T at 40°C for 15 min. When biotin labeled RNA is used the hybridized RNA should be stained with a

streptavidin-phycoerythrin in 6xSSPE-T at 40°C for 5 min. The arrays are read using a scanning confocal microscope made by Molecular Dynamics (commercially available through Affymetrix, Santa Clara, CA). The scanner uses an argon ion laser as the excitation source, with the emission detected by a photomultiplier tube through either a 530 nm bandpass filter (flourescein) or a 560 nm longpass filter (phycoerythrin). Nucleic acids of either sense or antisense orientations may be used in hybridization experiments. Arrays for probes with either orientation (reverse complements of each other) are made using the same set of photolithgraphic masks by reversing the order of the photochemical steps and incorporating the complementary nucleotide.

3. Quantitative analysis of hybridization patterns and intensities.

Following a quantitative scan of an array, a grid is aligned to the image using the known dimensions of the array and the corner control regions as markers. The image is then reduced to a simple text file containing position and intensity information using software developed at Affymetrix (available with the confocal scanner). This information is merged with another text file that contains information relating physical position on the array to probe sequence and the identity of the RNA (and the specific part of the RNA) for which the oligonucleotide probe is designed. The quantitative analysis of the hybridization results involves a simple form of pattern recognition based on the assumption that, in the presence of a specific RNA, the perfect match (PM) probes will hybridize more strongly on average than their mismatch (MM) partners. The number of instances in which the PM hybridization is larger than the MM signal is computed along with the average of the logarithm of the PM/MM ratios for each probe set. These values are used to make a decision (using a predefined decision matrix) concerning the presence or absence of an RNA. To determine the quantitative RNA abundance, the average of the difference (PM-MM) for each probe family is calculated. The advantage of the difference method is that signals from random cross-hybridization contribute equally, on average, to the PM and MM probes, while specific hybridization contributes more to the PM probes. By averaging the pairwise differences, the real signals add constructively while the contributions from cross-hybridization tend to cancel. When assessing the differences between two different RNA samples, the hybridization signals from side-by-side experiments on identically synthesized arrays are compared directly. The magnitude of the changes in the average of the

difference (PM-MM) values is interpreted by comparison with the results of spiking experiments as well as the signals observed for the internal standard bacterial and phase RNAs spiked into each sample at a known amount. Data analysis programs, such as those described in US Patent No.08/828,952 perform these operations automatically.

CONCLUSION

The inventions herein provide a pool of unique nucleic acid sequences which are complementary to approximately 6500 specific known murine genes. These sequences can be used for a variety of types of analyses.

The above description is illustrative and not restrictive. Many variations of the invention will become apparent to those of skill in the art upon review of this disclosure. The scope of the invention should, therefore, be determined not with reference to the above description, but instead be determined with reference to the appended claims along with their full scope of equivalents.

09/396196

TABLE 1

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TABLE 1

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TABLE 1

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TABLE 1

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42882	aatcggctggacaccttcctgagcc	U27295
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TABLE 1

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TABLE 1

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86167	ccatctttgctccgtgatgtcttcc	U75506
86168	ttgctccgtgatgtcttccacacga	U75506
86169	atcttatttcactaaaagtctcatc	AA106406
86170	tcttatttcactaaaagtctcatca	AA106406
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86173	cttattatcacccatatagtgctaa	AA106406
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86224	tctgacctctacaggcaaaattagg	D21099
86225	gcaccccctgtggtgcacatatatgc	D21099
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86227	cacgccaaacacatgaagttacaaa	D21099
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TABLE 1

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1025	cctcatagaggccctgaagatgaga	M96430	43629	acaccagccacagggcctggtccag	AA030302	86233	caacgtatctgcagtctcgctacta	AA106486
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TABLE 1

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TABLE 1

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43825	tgcagcgccagttacaacaaagcct	AA023081
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TABLE 1

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86830	aggatcttcgggacacctttgcagc	
86831	tggagctactgccttgcccccagga	
86832	ggagctactgccttgcccccaggag	
86833	ggcacccaagaaggccaagagaagg	
86834	gcacccaagaaggccaagagaaggg	
86835	taagacatggcacccaagaaggcca	
86836	aagacatggcacccaagaaggccaa	
86837	agacatggcacccaagaaggccaag	
86838	gacatggcacccaagaaggccaaga	
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86840	catggcacccaagaaggccaagaga	

TABLE 1

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1638	acttaccagttcaggctgctgtatg	L30104
1639	tcattccaggcccttgacggctttc	L30104
1640	ggctttcccggatgcacgttgacca	L30104
1641	cccggatgcacgttgaccagcggcg	L30104
1642	ttcttggctcacacggaacaactcc	L30104
1643	tttgatcccatcttgcaacgagtgg	L30104
1644	gacgccagcagagtcactttgacag	M30514
1645	tggaccgagtctgcttcctagccat	M30514
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1663	ctttgccagactgaggccaaccaat	M30514
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1700	ttttccatacagtgcttttgtacct	D76440

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86845	ggcacccaagaaggccaagagaagg	
86846	gcacccaagaaggccaagagaaggg	
86847	taagacatggcacccaagaaggcca	
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86850	gacatggcacccaagaaggccaaga	
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86906	gtgcactgcccagagttaggagacg	X97650
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TABLE 1

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TABLE 1

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TABLE 1

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TABLE 1

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TABLE 1

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TABLE 1

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TABLE 1

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TABLE 1

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TABLE 1

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88197	cttcagacctaatcacagctcagcg	L29006
88198	tcacagctcagcgtctgctccttca	L29006
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TABLE 1

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2997	agatcttcatgagtgctggtgtttt	U04055	45601	gaagcctcgataccaacgaggtgac	U50595	88205	gcttgagcccagttgaaggatttta	L29006
2998	atcttcatgagtgctggtgttttcg	U04055	45602	agaccaggatcttccgagtactggg	U50595	88206	ccctatctcatcatttagcacttat	L29006
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TABLE 1

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TABLE 1

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TABLE 1

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TABLE 1

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45944	caccaggagctaccaccgctgactg	X54352
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46004	actctataaagaccttgttgtgagc	AA064208

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09/396196

TABLE 1

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TABLE 1

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TABLE 1

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TABLE 1

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TABLE 1

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4012	agaccaactctttcccagaatcgct	M77174

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TABLE 1

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TABLE 1

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89419	gttcctcacagctcagcagagctgg	AA109998
89420	cacagctcagcagagctggaagaaa	AA109998
89421	gaaagtcagtcaacactgaggaacc	AA109998
89422	agtcagtcaacactgaggaaccaca	AA109998
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TABLE 1

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4219	ggatcatccacatgctgttcggggt	W49003	46823	cgacattgtcgtctgctgaggtagt	AA023922	89427	gtgactgggcttccagatggagccg	AA109999
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4221	gggtcagcctgagccttttcaacca	W49003	46825	atgatatcttttgaccttctcagtg		89429	cccacaggcactgagtacgagactc	AA109999
4222	atatctttgggtcatcatggagcgt	W49003	46826	ttgaaacttctcgttttggatgtag		89430	gagtacgagactctcactgctaagt	AA109999
4223	tctttgggtcatcatggagcgtacg	W49003	46827	tgatatcttttgaccttctcagtga		89431	gagactctcactgctaagtttcact	AA109999
4224	tacggccaatgttcaccatagggaa	W49003	46828	gatatcttttgaccttctcagtgat		89432	actctcactgctaagtttcacttcg	AA109999
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TABLE 1

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4287	cccacatgcatgcagtacctgtaga	J03293	46891	atctgcgctccgaactatcccaaca	AA028322	89495	tgtactaacttctccagaggcaagt	AA109910
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4617	cgtcatgtattgccatccgagagtc	W49163
4618	catgtattgccatccgagagtcggc	W49163
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4622	ttgacttcttcataggggatgaagc	W49163
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4624	agctcggctacgcaggcaacactga	W49163

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47165	cctcctgagccatgaagagaatata	Z33637
47166	ttggactcccagtttatagttgtct	Z33637
47167	atgagtaccctgccagcacaggagg	Z33637
47168	tcccttcaatctacccagtctctgg	Z33637
47169	caatctacccagtctctggtgccaa	Z33637
47170	gttctgctccaggaactctgccctg	Z33637
47171	gttcagggcagtgtgtcttttactt	Z33637
47172	ttggaagtccccagtcctgactcta	Z33637
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47174	agtcctgactctataccagtatggc	Z33637
47175	gactctataccagtatggctgagcc	Z33637
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47177	cccaaatccttgtcattgttttgat	Z33637
47178	tggccatgcttgcacccagaaattt	Y00516
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47189	cactgccaataaacaactatttaag	Y00516
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47225	tcaatgcctgcgtaaggaatgatgc	AA024208
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47227	ttcttggcacaaccgtatattccgg	AA024208
47228	gcacaaccgtatattccggagtatt	AA024208

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89769	ccatttgtccaatacatttgctttt	Z11997
89770	tttataagacccaaactcattcatt	Z11997
89771	ataagacccaaactcattcattaat	Z11997
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89783	tttgcccttctatctagatatggat	Z11997
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89786	catgctcaccaacagacagaaggtt	D49730
89787	tcctagataactccagacataagat	D49730
89788	ataactccagacataagatactcaa	D49730
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89802	ttagtacctagtcggtgaatccaga	D49730
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89804	taagtctcctagataactccagaca	D49730
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89828	gggcttcaggacttcacctccaaag	U57344
89829	accaagagcccacttgagggtctct	U57344
89830	aagagcccacttgagggtctctcaa	U57344
89831	ccacttgagggtctctcaagagcaa	U57344
89832	aagacaaagcctccagttcctgcac	U57344

TABLE 1

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4630	agccacagttcattattccgtcatg	W49163
4631	ctaaggctcagactctccaaggtgg	W48965
4632	ggtgatctctacgtgggaaagacta	W48965
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4637	ccctacagcctccagatctgggaca	W48965
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4691	cggctgagtttcgtctcatggaagc	W48968
4692	cgagcatgcgagttcaccgaagctg	W49178

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47296	ccaaaatgtcagagtcccaggacat	Z23143

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89897	cccttttcaggactcctggagtccag	AA110061
89898	ttggtcttgctcacatatcccaagt	AA110061
89899	gactcctggagtccaggggccttgt	AA110061
89900	ctcacatatcccaagtcatttcgga	AA110061

TABLE 1

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4694	ctcagctttctccatctgccctgtg	W49178	47298	ttaaactctgacgtcagatacttgt	Z23143	89902	cccaagtcatttcggaagttgatgg	AA110061
4695	cagctttctccatctgccctgtgta	W49178	47299	ggctgccaggctccaaacagccatg	AA024290	89903	tcatttcggaagttgatgggaacag	AA110061
4696	tccatctgccctgtgtactccagtg	W49178	47300	tgccaggctccaaacagccatgctt	AA024290	89904	tgctgcttttgacccggcggctgaa	AA110061
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90168	cttcatcccagggtttctagtcctg	
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TABLE 1

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4997	cagtgtctttgttgccaagatgagg	L28835	47601	agcaggccctgcactctcgctttct	X15267	90205	attctccatgctcaaacactgaaaa	AA110466
4998	gcccgctctgcaaatgaactggagg	L28835	47602	ggccctgcactctcgctttctggag	X15267	90206	taacaaccttcacgcacttatggct	AA110466
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5010	agtcagcagcggcattttgtcagcc	L28835	47614	gatcagccgatgacaaattgtgagc	AA027544	90218	tgctcaaacactgaaaataagagca	AA110466
5011	tctggcccagacgattgagaagaag	L28835	47615	agagaaagctggccgccatgtgcgc	AA027544	90219	gttttgagccactatattaagactg	AA110466
5012	tgtcacaggtccactgagtcactac	L28835	47616	gtgttgactgtctgcaacgatgaaa	AA027544	90220	tttgagccactatattaagactgct	AA110466
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TABLE 1

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5036	agtctgcgtgacttcttaggaaata	W49330	47640	cagtattgaccttatactagaaggc	X66091	90244	tcctggacactgtcatcaagtacct	D38614
5037	cgatcatcacacattctagtgcgta	W49330	47641	tattgaccttatactagaaggcgag	X66091	90245	tggacactgtcatcaagtacctgcc	D38614
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47840	gacctgtcctgtctgtctgaacctg	AA027694

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TABLE 1

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TABLE 1

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5307	ccactacaagctgcacggagtgaac	AA041982	47911	tctgaattttccagctcctcattgg	U03457	90515	atcttgtgtgtggttactgcctaat	D88315
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5371	gaaccgcaacatcaaccgcctgcag	X12789	47975	accaagaatgccgccatcgtagtgg	AA028220	90579	gtaattcagcgcgtttacagtcttt	AA110735
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TABLE 1

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TABLE 1

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TABLE 1

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TABLE 1

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91393	ttcttttccagataacactgcactt	AA116282
91394	tgcacttgcgtctctctgcctgcat	AA116282
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TABLE 1

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6191	caaccactctggcgactacgaatgt	W50102	48795	tcgtggtgcgcgacgagaatagcag	AA030256	91399	gtctttgtttgttcttctagcaaga	AA116282
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TABLE 1

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TABLE 1

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TABLE 1

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TABLE 1

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TABLE 1

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92000	gcacagcccgcttggtcttacaagt	AA117151
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92005	cctgacaacactgaattcaaacaaa	AA117151
92006	aagtgtgacctctatgtgagagcta	AA117151
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96T396T/60

TABLE 1

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6807	ccactgccgctgagtgtctccggca	W50138	49411	catcactacacgcagaatcatggag	AA031244	92015	gtctcaagaagcactaggcacagcc	AA117151
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TABLE 1

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TABLE 1

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6940	ctgactgccacctgaagggcaactc	X60103	49544	tcatgctctacacccgcaagtttga	AA030865	92148	ccactcactgtgaatttgcttacct	AA117227
6941	gcaactccaagtatcccaactgtga	X60103	49545	ccctcagctcgacttgaaacggcaa	X53028	92149	actcactgtgaatttgcttacctgc	AA117227
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TABLE 1

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TABLE 1

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TABLE 1

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TABLE 1

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